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Cultural and PCR-based detection of *Septoria musiva* in inoculated hybrid poplar stems

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Summary

The fungal pathogen *Septoria musiva* can be difficult to isolate from cankers that result from its colonization of poplar stems, and its persistence in these cankers has not been well studied. In order to compare cultural and polymerase chain reaction (PCR)-based assays for detection of *S. musiva* in cankers, stems of susceptible hybrid poplar clone NC11505 were wounded and inoculated in August 2003. At 8, 16, 24 and 32 weeks after inoculation (October and December 2003, February and April 2004, respectively), 110 inoculated stems (plus controls) were harvested and a semiselective culture medium was used in attempts to detect the pathogen in bark and wood. Six chips of bark and six chips of underlying wood from one half of each canker were incubated on the semiselective medium for 2 weeks until pycnidia and conidia of *S. musiva* could be identified. The number of positive cankers and positive chips (out of six attempts per tissue per canker) was recorded. The remaining halves of cankers from subsets of 70 inoculated stems (plus controls) of those harvested in October 2003 and April 2004 were tested using a PCR-based assay. Three chips of bark and three chips of underlying wood were ground, and DNA was extracted and then amplified using *S. musiva*-specific primers designed from the internal transcribed spacer (ITS) region of nuclear rDNA repeats. The number of positive cankers and positive chips (out of three attempts per tissue per canker) was recorded. For both assays, the number of positive cankers and the number of positive chips per canker decreased with time. Using either assay, however, the pathogen was still detected from at least 49% of cankers at 32 weeks after inoculation.

1 Introduction

Mycosphaerella populorum is a serious canker pathogen of poplar (*Populus*) species and hybrids. The fungus is usually encountered in its anamorphic state, *Septoria musiva*, which produces characteristic oblong conidia in pycnidia. Infection and colonization of the trunk and branches can lead to stem distortion, breakage and death (Bier 1939; Waterman 1954; Ostry and McNabb 1985; Spielman et al. 1986). Its presence in eastern and central North America has precluded the use of susceptible, but otherwise highly productive poplar clones in short-rotation intensive culture (SRIC) poplar plantations. In the western United States and western Canada, where the pathogen is reportedly absent (Newcombe et al. 1995; Callan 1998), SRIC plantations can be valuable sources of fibre and lumber (Ondro 1991; Johnson 2000; Rice 2000; Van Oosten 2000).

Detection of *S. musiva* from stem tissues of poplar can be difficult. Pycnidia and pseudothecia of the pathogen are rarely found on older cankers in comparison with the abundance of other fungi and their fruiting structures (Bier 1939; Waterman 1946, 1954). Furthermore, isolation of the pathogen from naturally infected stem tissues or from sites of inoculation can be relatively infrequent (Waterman 1954; Schipper 1976; Spielman et al. 1986; Lo et al. 1995; Stanosz and Stanosz 2002). Use of a fungicide-amended culture medium can enhance identification of *S. musiva* from cankers (Stanosz and Stanosz

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2002). However, we continue to sometimes experience difficulty isolating this pathogen from even relatively young cankers typical of those attributed to *S. musiva*. This difficulty could indicate lack of persistence after canker initiation, or merely the inability to detect it in the presence of interfering organisms or compounds.

In an effort to quantify the persistence of *S. musiva* in young stem cankers, a field study was conducted during 2003 and 2004. Both a cultural and a polymerase chain reaction (PCR)-based assay were used to detect the pathogen from the bark and wood of inoculated, susceptible poplar clone NC11505. The objectives of this study were to determine whether: (i) frequency of *S. musiva* detection from cankered tissues decreases with increasing time from inoculation; (ii) frequency of detection is affected by the tissues sampled and (iii) efficiency of detection is affected by the assay method used.

2 Materials and methods

2.1 Field design and establishment

Two hundred and sixty-eight cuttings of susceptible clone NC11505 (*Populus maximowiczii* × *P. trichocarpa*) were established in the summer of 2002 on a spacing of 1.2 m by 1.8 m into each of two plots at the University of Wisconsin-Madison West Madison Agricultural Research Station (total of 536 trees). Soil at the site is well-drained Plano silt loam that was previously planted in alfalfa. Each tree was mulched with a 0.91 × 0.91 m square of perforated black plastic (Vispore tree mats; Treessentials Co., Mendota Heights, MN, USA). Subsequent maintenance included both mechanical and chemical (glyphosate) management of competing vegetation. Sixty-seven trees within each of the two plots were randomly assigned to each of four harvest dates at 8-week intervals following inoculation in August 2003 (October and December 2003, February and April 2004). Trees within each group of 67 were further randomly assigned to five replicates of 13 or 14 trees.

2.2 Inoculation

Inoculum was produced from a single-conidial isolate of *S. musiva* (isolate 92-49A, DAOM 229444) obtained from a hybrid poplar leaf lesion. This isolate induced cankers after inoculation of poplar stems in previous studies (Maxwell et al. 1997; Stanosz and Stanosz 2002; Weiland et al. 2003). Conidia (1.7×10^6 spores/ml) stored in sterile water at −80°C were streaked in three equally spaced lines on the surface of 20 ml of malt extract agar (MEA; 10 g malt extract, 20 g agar, 1000 ml water) in 84-mm-diameter Petri dishes and allowed to grow for 2 weeks at 21°C under continuous fluorescent light. Plugs of inoculum (5 mm diameter) then were cut from colony margins. Eleven trees per replicate in each plot were inoculated by removing the fourth or fifth fully expanded leaf and placing a plug of inoculum on the resulting wound with the mycelium side towards the stem (Mottet et al. 1991; Maxwell et al. 1997). The remaining two or three trees per replicate in each plot were treated as controls by applying a sterile MEA plug onto the fresh wound. Dates of inoculation were 16–17 and 18–19 August 2003 for plots 1 and 2 respectively.

2.3 Harvests and canker preparation

At each of the four harvest dates, five replicates of 11 inoculated trees and two or three control trees (total of 67 trees) were harvested from each plot. A 15-cm-long segment of each stem, centered on the inoculation point, was cut and stored in a plastic bag at 5°C for up to 36 h. Upon removal from refrigeration, canker presence or absence for each stem was recorded and stem segments were surface-disinfested for 30 s in 95% ethanol. Each canker

was divided longitudinally in half and then a sample of symptomatic bark and a sample of the underlying wood were aseptically excised from each half. Each sample was oriented along the longitudinal axis of the stem, on either side of the inoculation point, and extended approximately 2 mm beyond the visible necrotic proximal margin of the canker. Comparable samples were excised from each control stem. One bark sample and the associated, underlying wood sample from each stem was randomly selected to be plated on semiselective medium. The remaining samples were frozen at −20°C and a subset was analysed at a later date using the PCR-based assay (described below).

2.4 Cultural assay

Each bark sample was divided into six equally sized chips (2.5 × 5 mm) and aseptically transferred to a Petri plate containing *S. musiva* medium (SMM), an amended V8 juice agar that was developed to facilitate identification of this fungus from poplar cankers (Stanosz and Stanosz 2002). Similarly, the associated underlying wood sample was divided into six chips and transferred to a plate containing SMM. The plates then were placed in an incubator at 21°C, 30 cm below continuous fluorescent light as described by Krupinsky (1989) for 2 weeks, and then examined for the presence or absence of the pathogen on each chip. The presence of *S. musiva* was determined on the basis of pycnidia and conidia as described by Sivanesan (1990).

2.5 PCR-based assay

Bark and wood samples collected in October 2003 and April 2004 from five to seven inoculated trees and one control tree in each replicate of each plot were tested using the PCR-based assay. Samples to be tested were selected randomly from each replicate. Bark and wood were thawed, and each was divided into three equally sized chips (5 × 5 mm) for DNA extraction. DNA was extracted by a modified procedure from Cubero et al. (1999). Briefly, each chip of bark or wood was cut into smaller pieces (<1 mm^2) and placed into a separate 1.5-ml microfuge tube with 250 μl of CTAB extraction buffer [1% w/v CTAB, 1 M NaCl, 100 mM Tris, 20 mM ethylenediaminetetraacetic acid (EDTA), 1% w/v polyvinylpyrrolidone] and sterile, acid-washed sand. Tissues were ground briefly with a sterile Kontes pestle (Vineland, NJ, USA) and an electric drill and then incubated at 70°C for 30 min. One volume of chloroform : isoamyl alcohol (24 : 1) was added to the mixture, which was then vortexed briefly and centrifuged at 10 000 ***g*** for 7 min. One hundred and fifty microlitre of supernatant was transferred to a new 1.5-ml microfuge tube, and two volumes of precipitation buffer (1% w/v CTAB, 50 mM Tris, 10 mM EDTA, 40 mM NaCl) were added, and then mixed for 2 min by inversion. The mixture then was centrifuged at 14 000 ***g*** for 15 min. The supernatant was aspirated, and the DNA pellet was resuspended in 350 μl of 1.2 M NaCl. One volume of chloroform : isoamyl alcohol (24 : 1) was added, the mixture was vortexed briefly, then centrifuged at 8000 ***g*** for 5 min. The supernatant was transferred to a new 1.5-ml microfuge tube and 0.6 volume of isopropanol was added. This was vortexed briefly and then incubated at −20°C for 15 min before centrifuging at 14 000 ***g*** for 20 min. The supernatant was aspirated, the DNA pellet was washed with 70% ethanol, and then dried at room temperature. The DNA pellet was resuspended in 35 μl of 10 mM Tris and stored at −20°C.

Total genomic DNA was used as template for PCR amplification. Amplification was performed in 25 μl reactions containing 1X buffer, 3 mM $MgCl_2$, 0.6 mM dNTPs, 1 U *Taq* DNA polymerase, 0.1 μM of primers Smusf and Smusr (5′-AGA-GAAGCGTGGCGCCCC and 5′-CCAGGCTTGAGTGGTTGTACT, respectively) to detect *S. musiva* (Feau et al. 2005), and 2 μl of template DNA. Primers were designed from the internal transcribed spacer (ITS) region of nuclear ribosomal RNA gene (rDNA)

repeats. Primers were specific for *S. musiva* when tested against the related poplar pathogens *S. populicola* and *S. populi*, as well as 12 additional *Septoria* species and 17 other fungal species obtained from stems or leaves of poplars (FEAU et al. 2005). Amplification was performed in a Biometra T-gradient Thermal Cycler (Göttingen, Germany) using touchdown PCR with the following temperature profile: one cycle of 3 min at 94°C; 30 cycles of 45 s at 94°C; 45 s at an annealing temperature that decreased 0.67° C/cycle from an initial temperature of 68°C and 1 min at 72°C. The PCR products were separated by electrophoresis on 0.7% agarose gels in 0.5X TBE buffer. Gels were stained with ethidium bromide and photographed under UV light. The presence of *S. musiva* was determined on the basis of amplification of a 330 bp fragment. The identity of 20 randomly selected DNA fragments from both bark and wood in October 2003 and April 2004 was confirmed using an AlkPhos Direct Labeling and Detection System with CDP Star (Piscataway, NJ, USA), a non-radioactive, dot blot method for DNA hybridization.

2.6 Statistical analyses

The mean frequency of pathogen detection per replicate was analysed by two-way ANOVA for effects of assay method, date of harvest, plot and type of tissue. Mean frequency data were converted into proportions (mean percentage of positive cankers/100) and transformed by the arcsine of the square root before analyses. Tukey's simultaneous tests were used to conduct pairwise comparisons on the effects of time on pathogen detection. In addition, the number of positive chips per canker was analysed using the chi-square test of independence to determine if the number of positive chips per canker was independent of date of harvest or tissue. Finally, data from the cankers common to both experiments (calculated from five replicates of five to seven trees in each plot) were analysed to allow direct comparison of the effectiveness of assay method on pathogen detection. Analyses were performed using MINITAB Statistical Software (release 14.1; Minitab Inc., State College, PA, USA).

3 Results

3.1 Canker characteristics

Cankers were visible on the surface of inoculated stems approximately 1 month after inoculation. The cankers developed to closely resemble those attributed to natural infection of poplars by *S. musiva* in the field. The bark surface immediately overlying the cankers was sunken, darkly discoloured greenish-brown to black and necrotic. Bark on the face of the canker often split longitudinally. The canker margin on some of the trees was swollen due to production of callus. When the outer bark was carefully removed, the canker usually extended beyond the margin visible at the bark surface. Inner bark and wood were discoloured reddish-orange to black and discolouration often extended into the pith. At the third and fourth harvest dates in February and April, cankers had developed distinctive orangish-green margins. By the final harvest date in April, pycnidia and conidia of the pathogen were observed in the cankers on approximately 25% of the inoculated stems. No control trees in either plot developed cankers.

3.2 Cultural assay

The mean percentage of cankers from which *S. musiva* was isolated (positive cankers) was greatest in October (92% for bark, 98% for wood) and had decreased approximately 40% by the sampling date in April (56% for bark and wood; Table 1). The mean percentage of

Table 1. **Frequency of detection of *Septoria musiva* from wounded and inoculated[1] poplar stems that were tested using a cultural assay[2]**

		Cankers positive (%)		Number of cankers with 0–6 positive chips[4]						
Date of harvest	Tissue	Mean (%)[3]	SE	0	1	2	3	4	5	6
October	Bark	92	2.1	9	5	12	11	17	25	31
	Wood	98	1.2	2	4	4	14	9	23	54
December	Bark	73	4.1	30	13	19	24	12	10	2
	Wood	73	3.6	30	9	18	13	16	10	14
February	Bark	72	3.2	31	20	30	13	12	4	0
	Wood	71	3.3	32	12	17	17	17	11	4
April	Bark	56	3.8	48	16	15	10	13	4	4
	Wood	56	4.0	48	15	9	15	8	10	5

[1]Trees of clone NC11505 were inoculated in August by removing the fourth or fifth fully expanded leaf and placing a plug of medium bearing mycelium on the resulting wound.
[2]Six chips of bark and wood from each canker were incubated on plates of *S. musiva* medium at 21°C, 30 cm from continuous fluorescent light.
[3]Mean percentage of cankers positive was calculated from 10 replicates of 11 trees. Two-way analysis of variance indicated an effect of date of harvest ($p < 0.001$), but no effect of tissue ($p = 0.267$) or interaction ($p = 0.100$).
[4]Number of cankers with 0–6 positive chips per canker. Chi-square analysis indicated that the number of positive chips per canker was not independent of date of harvest ($p < 0.001$) or tissue in October ($p = 0.007$), December ($p = 0.034$), or February ($p = 0.027$). In April, the number of positive chips per canker was independent of tissue ($p = 0.357$).

positive cankers, as detected from bark tissues, decreased the most in the periods from October to December 2003 (21%), and from February to April 2004 (21%). Likewise, the mean percentage of positive cankers, as detected from wood tissues, decreased the most in the periods from October to December 2003 (26%) and from February to April 2004 (20%). Except for the comparison of data from December to February (for which detection was not significantly different, $p = 0.927$), differences in detection between dates of sampling (e.g. October–December) were always significant (values of $p < 0.005$). Analysis of variance indicated an effect of time ($p < 0.001$), but no effect of tissue ($p = 0.267$) or interaction ($p = 0.100$) on the mean percentage of positive cankers at each of the four harvest dates. Because ANOVA indicated that there was no plot effect ($p = 0.171$), pooled data are presented.

Most cankers yielded five or six positive bark or wood chips in October (Table 1). In contrast, the following three harvest dates saw a decrease in the number of cankers yielding five or six positive bark or wood chips. Although the number of positive chips per canker was not independent of tissue in October, December, or February, results from bark were similar to results from wood. Chi-square analysis indicated that the number of positive chips per canker was not independent of date of harvest ($p < 0.001$) or of tissue in October ($p = 0.007$), December ($p = 0.034$) and February ($p = 0.027$). However, the number of positive chips per canker was independent of tissue ($p = 0.357$) in April. Because chi-square analysis indicated that the number of positive chips per canker was independent of plot ($p \geq 0.116$), pooled data are presented.

Fungi other than *S. musiva* were observed growing from the diseased tissues plated on SMM at all harvest dates. Furthermore, more fungi were observed growing from plated bark tissues than from wood. Although their frequency was not quantified, species of *Epicoccum*, *Fusarium*, *Alternaria* and *Cytospora* were more abundant at the fourth harvest date than at the first when many chips yielded only *S. musiva*. Isolates of *Cytospora* spp., in

Table 2. **Frequency of detection of *Septoria musiva* from wounded and inoculated[1] poplar stems that were tested using a polymerase chain reaction (PCR)-based assay[2]**

		Cankers positive (%)		Number of cankers with 0–3 positive chips[4]			
Date of harvest	Tissue	Mean (%)[3]	SE	0	1	2	3
October	Bark	86	4.8	9	8	23	24
	Wood	56	5.0	28	13	15	8
April	Bark	66	5.8	23	12	17	17
	Wood	49	5.6	35	12	14	8

[1]Trees of clone NC11505 were inoculated in August by removing the fourth or fifth fully expanded leaf and placing a plug of medium bearing mycelium on the resulting wound.
[2]DNA was extracted from three chips of bark and three chips of wood from each canker, and the internal transcribed spacer (ITS) region of *S. musiva* was amplified using the method provided in the text.
[3]Mean percentage of cankers positive was calculated from 10 replicates of five to seven trees. Two-way analysis of variance indicated an effect of date of harvest ($p = 0.016$), an effect of tissue ($p < 0.001$), but no interaction ($p = 0.144$).
[4]Number of cankers with 0–3 positive chips per canker. Chi-square analysis indicated that the number of positive chips per canker was not independent of date of harvest for bark ($p = 0.031$), but was independent of date of harvest for wood ($p = 0.881$). The number of positive chips per canker was not independent of tissue in October ($p < 0.001$), but was independent of tissue in April ($p = 0.111$).

particular, were more frequently observed on plates in April than from the three previous harvest dates.

3.3 PCR-based assay

The mean percentage of positive cankers for *S. musiva* in bark decreased approximately 20% from October (86%) to April (66%; Table 2). The mean percentage of positive cankers based on testing of wood was comparatively lower in October (56%) but decreased relatively little by April (49%). Analysis of variance indicated an effect of date of harvest ($p = 0.016$) and tissue ($p < 0.001$), but no interaction ($p = 0.144$) on the mean percentage of positive cankers at the two harvest dates. Because ANOVA indicated that there was no plot effect ($p = 0.782$), pooled data are presented.

Most cankers yielded two or three positive bark chips in October (Table 2). In contrast, most cankers from the same harvest period yielded one or no positive wood chips. By April, the number of cankers that yielded two or three positive chips had decreased for both bark and wood tissues, but again the number of cankers yielding two or three positive chips was greater for bark than for wood. Chi-square analysis indicated that the number of positive chips per canker was not independent of date of harvest for bark ($p = 0.031$), but was independent of date of harvest for wood ($p = 0.881$). In October, the number of positive chips was not independent of tissue ($p < 0.001$), but was independent of tissue by April ($p = 0.111$). Because chi-square analysis indicated that the number of positive chips per canker was independent of plot ($p \geq 0.105$), pooled data are presented.

3.4 Comparison of cultural and PCR-based assays

Congruence of results obtained (i.e. the number of cankers giving the same result using both assay methods) decreased from October to April. Of the 64 cankers that were positive using either method in October, 59 (92%) were positive for both methods. Of the 56

Table 3. **Frequency of detection of *Septoria musiva* from a subset of wounded and inoculated[1] poplar stems that were tested using cultural and polymerase chain reaction (PCR)-based assays[2]**

		Method			
		SMM		PCR	
Date of harvest	Tissue	Mean (%)[3]	SE	Mean (%)	SE
October	Bark	93	2.5	86	4.6
	Wood	97	2.1	56	5.1
April	Bark	56	6.1	66	5.8
	Wood	51	5.6	49	5.6

[1]Trees of clone NC11505 were inoculated in August by removing the fourth or fifth fully expanded leaf and placing a plug of medium bearing mycelium on the resulting wound.
[2]Six chips of bark and six chips of wood from each canker were incubated on plates of *S. musiva* medium (SMM) at 21°C, 30 cm from continuous fluorescent light for the culture method. In the PCR-based assay, DNA from three chips of bark and three chips of wood from each canker was extracted, and the internal transcribed spacer (ITS) region of *S. musiva* was amplified using the method provided in the text.
[3]Mean percentage of cankers positive was calculated from 10 replicates of five to seven trees. Two-way analysis of variance indicated an effect of assay method ($p = 0.001$), date of harvest ($p < 0.001$) and tissue ($p = 0.003$) and interactions of method × date of harvest ($p < 0.001$), method × tissue ($p < 0.001$) and method × date of harvest × tissue ($p = 0.041$). No interaction of tissue × date of harvest was observed ($p = 0.842$).

cankers that were positive using either method in April, only 37 (66%) were positive for both.

The mean percentage of positive cankers (from the data set common to both detection assays) varied by both assay method and harvest date (Table 3). Using the cultural assay, frequencies of detection were similar from bark and wood in October, and again in April, and decreased approximately 40% during this interval. Although frequencies of detection with the PCR-based assay were generally similar to those obtained in the cultural assay, detection from wood was considerably lower than for bark in October. In addition, differences in the ability of each assay to detect the pathogen from each canker were observed. For the 140 canker samples (both bark and wood tissues) tested in October, *S. musiva* was detected in 94 samples with both methods, in 39 samples with the cultural assay only, in five samples using the PCR-based assay only and was not detected in two samples using either assay. For the 140 canker samples tested in April, *S. musiva* was detected in 57 cankers with both methods, in 19 cankers with the cultural assay only, in 25 cankers using the PCR-based assay only and was not detected in 39 cankers using either assay. Analysis of variance indicated an effect of method ($p = 0.001$), date of harvest ($p < 0.001$) and tissue ($p = 0.003$) on the mean percentage of positive cankers at the two harvest dates. Interactions of method × date of harvest ($p < 0.001$), method × tissue ($p < 0.001$) and method × date of harvest × tissue ($p = 0.041$) were also observed, but not tissue × date of harvest ($p = 0.842$). Because ANOVA indicated that there was no plot effect ($p = 0.365$), pooled data are presented.

4 Discussion

Survival of *S. musiva* in diseased, woody tissue throughout the fall, winter and spring following inoculation supports the characterization of *S. musiva* as a perennial canker pathogen (BIER 1939; WATERMAN 1954). Perennial cankers are characterized by a constant association of the pathogen with the host for many years, with growth by the host and

pathogen alternating depending on the season (Sinclair et al. 1987). *Eutypella parasitica*, for example, colonizes a certain amount of bark and wood on dormant sugar maple each year. The host then produces callus and a necrophylactic periderm around the lesion during the growing season. After the host becomes dormant, the pathogen is able to penetrate callus and the necrophylactic periderm to invade healthy tissue. In contrast, annual cankers such as those caused by *Fusarium solani* on sugar maple (Wood and Skelly 1964; Skelly and Wood 1966) develop and expand during a single dormant season. The stem is subsequently restored by the formation of callus and bark over the wound.

The non-linear decrease in the mean percentage of positive cankers from October 2003 to April 2004 could indicate that persistence is influenced by numerous interacting biotic and abiotic factors that change with time. The percentage decreased the most between October and December 2003 and February and April 2004, while remaining relatively stable from December 2003 to February 2004. During these intervals, there could be effects of host defence responses, changes in temperature, bark moisture and competition from other microorganisms. Future, longer term studies on the persistence of *S. musiva* in stem cankers may clarify these issues.

Lack of complete congruence in results obtained using the two assay methods indicates the need for caution in interpretation. For example, each method resulted in similar frequencies of pathogen detection among the population of cankers sampled in April. But because some cankers tested positive using one method and negative using the other, a negative result using a single method for an individual canker was not necessarily indicative of the absence of the pathogen in that canker. Because of this lack of congruence, results for each method also underestimated the overall frequency of pathogen presence among the population of cankers that were sampled at that time. Stenström and Ihrmark (2005) reported similar results in their comparison of PCR-based and cultural assays for detection of the pathogenic fungus *Lophodermium seditiosum* in pine needles. Although 28 of 90 needles from one location tested positive using at least one method, they found that each method detected the pathogen in only 19 needles, and only 10 needles tested positive for both the PCR-based and cultural assay.

The two assays used for detection of *S. musiva* offer different advantages and different possibilities for improvement. A positive result using the cultural method guarantees pathogen viability. Although detection using the semiselective medium requires 2 weeks to complete, this assay requires less labour, simple equipment, less costly materials and many samples can be plated in relatively little time. The numbers of bark or wood chips plated and numbers of cankers sampled can be increased relatively easily. In contrast, even though the PCR-based assay can yield results more quickly (DNA extraction and amplification can be completed in a single day), procedures are more complex and materials more expensive. The effort involved in effectively grinding tissues can be considerable, and various compounds in tree bark and wood can interfere with DNA extraction. Use of a ball mill for grinding tough tissues (Langrell 2005) and commercially available kits for cleaning extracts may improve PCR-based assay results.

Results of this study support the ability of *S. musiva* to colonize both bark and wood tissues. Previously, contradictory evidence existed on the stem tissues colonized by *S. musiva*. Krupinsky (1989) was able to isolate *S. musiva* from wood chips at the canker margin. Waterman (1954) was also able to isolate the fungus from wood, but did note that wood tissue from some cankers remained sterile when placed on malt agar. Recently, Stanosz and Stanosz (2002) more frequently isolated *S. musiva* from wood than from bark. Sivanesan (1990), however, comments that Septoria canker is confined to the bark of young stems and branches, but does not indicate whether the pathogen is also thus limited. Similarly, Zalasky (1978) found that hyphae of *S. musiva* colonized the cortex and phloem, but were delimited by phloem fibres and xylem.

The growth of *Cytospora chrysosperma* from diseased tissues in the present study is consistent with observations by previous investigators. Several researchers have reported the presence of *C. chrysosperma*, as well as other potentially pathogenic fungi, in cankers attributed to *S. musiva* (Bier 1939; Waterman 1946, 1954; Bowersox and Merrill 1976; Schipper 1976; Lo et al. 1995; Stanosz and Stanosz 2002; Zalasky et al. 1968). The presence of these, and other secondary fungi, might mask the presence of *S. musiva* and lead to misdiagnoses (Waterman 1954). Bier (1939), however, suggested that some cankers might result from the combined attack of *S. musiva* and *C. chrysosperma*, and Zalasky et al. (1968) considered *C. chrysosperma* as a successional fungal species on Septoria cankers. Due to the consistent association of *C. chrysosperma* with *S. musiva*, combined inoculations of the two fungi may be warranted in future studies of poplar canker aetiology.

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Résumé

Détection de Septoria musiva en culture et par PRC à partir de tiges de peupliers hybrides inoculés

Le champignon pathogène *Septoria musiva* est parfois difficile à isoler des chancres résultant de sa colonisation de tiges de peupliers, et sa persistance dans les chancres n'a pas été étudiée en détail. Afin de comparer des méthodes culturales et par PCR pour la détection de *S. musiva* dans les chancres, des tiges d'un clone sensible de peuplier hybride, NC11505, ont été inoculées après blessure en Août 2003. A 8, 16, 24 et 32 semaines après inoculation (Octobre et Décembre 2003, Février et Avril 2004 respectivement), 110 tiges inoculées (et des témoins) ont été prélevées et un milieu de culture semi sélectif a été utilisé pour détecter le pathogène dans le bois et l'écorce. Six morceaux d'écorce et six morceaux de bois sous-jacent prélevés sur la moitié de chaque chancre ont été incubés sur le milieu semi sélectif pendant deux semaines, jusqu'à ce que les pycnides et conidies de *S. musiva* puissent être identifiées. Le nombre de chancres positifs et de morceaux positifs (sur six isolements par tissu) ont été notés. La deuxième moitié des chancres d'un échantillon de 70 tiges inoculées (et des témoins) parmi celles prélevées en Octobre et Avril 2004 ont été testés par PCR. A partir de trois morceaux d'écorce et trois morceaux du bois sous jacent qui ont été broyés, l'ADN a été extrait puis amplifié en utilisant des amorces spécifiques de *S. musiva*, dans la région de l'ITS de l'ADN nucléaire ribosomal. Le nombre de chancres et de morceaux positifs ont été notés. Quelle que soit la méthode de détection, une décroissance du nombre de chancres positifs et du nombre de morceaux positifs par chancre a été observée avec le temps. Toutefois, le pathogène est encore détecté dans au moins 49% des chancres 32 semaines après inoculation, avec les deux méthodes.

Zusammenfassung

Nachweis von Septoria musiva in Hybridpappeln mit Isolierungen und PCR

Der Pilz *Septoria musiva* ist aus den Rindennekrosen, die er an Pappelstämmen verursacht, zum Teil schwer isolierbar und seine Persistenz in diesen Nekrosen wurde nur unzureichend untersucht. Zum Vergleich von zwei Nachweismethoden (Isolierungen und PCR) von *S. musiva* in Nekrosen wurden Stämme des anfälligen Hybridpappelklons NC11505 im August 2003 verletzt und inokuliert. Nach 8, 16, 24 und 32 Wochen (Oktober und Dezember 2003 sowie Februar und April 2004) wurden 110 inokulierte Stämme und zusätzliche Kontrollbäume entnommen und es wurde versucht, das Pathogen aus der Rinde und dem Holz mit einem semiselektiven Medium zu isolieren. Je 6 Probestücke aus der Rinde und dem darunter liegenden Holz aus einer Hälfte jeder Nekrose wurden für zwei Wochen auf dem Selektivmedium inkubiert bis Pyknidien und Konidien von *S. musiva* identifiziert werden konnten. Die Anzahl der positiven Nekrosen und der positiven Gewebeproben (bei 6 Isolierungen pro Nekrose) wurde registriert. Bei 70 Nekrosen an Stämmen, die im Oktober 2003 und April 2004 beprobt worden waren, wurde die zweite Hälfte der Nekrose mit einem PCR-Nachweis getestet. Zusätzlich wurden Kontrollbäume untersucht. Hierzu wurden je drei Stücke aus der Rinde und dem

darunter liegenden Holz zerkleinert, die DNA extrahiert und anschliessend mit für *S. musiva* spezifischen Primern aus der ITS-Region der nukleären rDNA amplifiziert. Auch hier wurde die Anzahl positiver Nekrosen und positiver Proben (bei jeweils drei Proben pro Gewebetyp) für jede Nekrose registriert. Bei beiden Nachweismethoden nahm die Anzahl positiver Nekrosen und positiver Proben pro Nekrose mit der Zeit ab. Das Pathogen konnte aber mit beiden Methoden 32 Wochen nach der Inokulation noch bei mindestens 49% der Nekrosen nachgewiesen werden.

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